Proliferation is necessary for both repair and mutation in transgenic mouse cells

Jason H. Bielas and John A. Heddle*

Biology Department, York University, 4700 Keele Street, Toronto, ON, Canada M3J 1P3

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Proliferating cells are often presumed to be more mutable than quiescent cells because they have less time to repair DNA damage before DNA replication. Direct tests of this hypothesis have been confounded by the need for cell division before a mutation can be detected. We have avoided this problem by showing that the Big Blue mouse cell line permits the dynamic quantification of both lesions and mutations in the complete absence of cell division. These cells carry the bacterial *lacI* gene in a λ shuttle vector. Mutant plaques recovered by *in vitro* packaging of the mouse DNA can arise from mutations sustained either in mouse cells or in the bacteria. The proportion of mutant phage contained within a mutant plaque can distinguish these two types of mutation. Mutations formed in mouse cells yield >90% mutant phage because both DNA strands are mutant. On the other hand, mutations formed in the bacteria from adducted DNA yield ≤50% mutant phage, because one of the DNA strands is wild type. Immediately after exposure to a test mutagen, ethylnitrosourea, all induced mutations were formed in the bacteria, but after approximately one cell division, the reverse was true and all mutations arose in the mouse cells. Only one-fifth as many mutations were recovered from quiescent cells and all arose in the bacteria, showing that the mouse cells made no mutations in the absence of proliferation. Surprisingly, the mouse cells did not repair any of the premutagenic damage during 4 days of quiescence. When these quiescent cells were induced to proliferate, however, both repair and mutation fixation ensued.

lacI | quiescent | *N*-ethyl-*N*-nitrosourea | cell division | Big Blue

It is evident that mutations can accumulate in actively dividing cells, but this is not as obvious for quiescent cells. Mutation is relatively easy to measure in proliferating cells and tissues, but mutation research in quiescent cells has been complicated by the nature of most mutational assays. Because the detection of mutations requires the formation of visible clones, a process that is itself dependent on DNA replication, it is almost impossible to know whether DNA replication is an essential step in mutation (1). Considering that most cells in our body are not actively dividing and spend large periods of time "resting" (2), the mutational events experienced by these cells are important and, in particular, may be involved in the origins of many human cancers.

Earlier work directed at elucidating the links among DNA synthesis, cell division, and mutation induction was conducted in arrested Chinese hamster ovary cells by O'Neill and colleagues (3–5). They treated cells that had been arrested by serum deprivation with the alkylating agent ethyl methanesulfonate (3) and compared the results with those obtained with proliferating Chinese hamster ovary cells. Mutation induction and expression were examined at the endogenous *hprt* locus at various times after treatment. The results from arrested cultures maintained in serum-free media for up to 18 days posttreatment indicated that (*i*) mutations were induced at the *hprt* locus during quiescence, and (*ii*) that the mutant frequency remained constant over time. This system was unable to accurately track repair of adducts because of the nature of the *hprt* mutational assay, which requires the initiation of cellular division and, thus, DNA replication for the detection of 6-thioguanine-resistant mutant clones. Lesions created by ethyl methanesulfonate treatment could have been repaired either during cell quiescence or early in the formation of 6-thioguanine-resistant clones. The inability to distinguish between these two mutational events made it impossible to unravel the link among DNA replication, mutation, and repair. Today, with the advent of transgenic cell lines, it is possible to reexamine the role of proliferation on both mutation and repair in mammalian cells.

The transgenic Big Blue mouse cell line created by Stratagene uses a λ shuttle vector carrying *lacI* as a mutational target gene. What makes this an ideal system for studying the relationship between proliferation and mutation is that after genomic DNA isolation, the *lacI* transgene can be retrieved and assayed for mutation from both proliferating and quiescent cells. Furthermore, the *lacI* system provides a means for quantifying mutation fixation, repair, and adduct stability in division-arrested and proliferating cultures, as this system permits the quantification of both premutagenic DNA adducts and mutations (6).

Mutant plaques arise primarily in one of two ways: (*i*) from cellular mutations that arise in the cell line, and (*ii*) from bacterial mutations that arise from DNA damage that become fixed in the bacterium as a mutation (Fig. 1). Although mutations also can occur as a result of errors introduced during phage replication, these mutations are very rare and only those few that occur during the first few rounds of replication produce a detectable mutant plaque (6). Initially, cellular and bacterial mutations are indistinguishable from one another, as they both form blue plaques (6). However, the origin of the mutation can be discovered by coring and replating the blue mutant plaque. Mutations arising in mammalian cells should contain only mutant phage, although in practice there are a few nonmutant contaminants. Mutations arising in the bacteria yield a mosaic mutant plaque, containing ≤50% mutant phage.

Mutation fixation, adduct stability, and repair in quiescent and proliferating Big Blue mouse cells were investigated after treatment with ethylnitrosourea (ENU) by classifying the *lacI* mutations as cellular or bacterial. Because bacterial mutations are representative of adducts localized on the *lacI* transgene, any decline observed in their frequency would signify adduct removal. Adduct removal by repair would decrease the number of mutant *lacI* plaques recovered, whereas mutation fixation would increase the proportion of cellular *lacI* mutations. By coring and replating all *lacI* mutant plaques, it is possible to examine the fate of premutagenic adducts on the *lacI* transgene in both dividing and nondividing Big Blue mouse cells after treatment.

Materials and Methods

Big Blue Mouse Embryonic Cell Line. The Big Blue mouse cell line was established from embryonic cells, containing tandem mul-

Abbreviations: ENU, ethylnitrosourea; AGT, O^6-alkylguanine-DNA alkyltransferase.

See commentary on page 11137.

*To whom correspondence should be addressed. E-mail: jheddle@yorku.ca.

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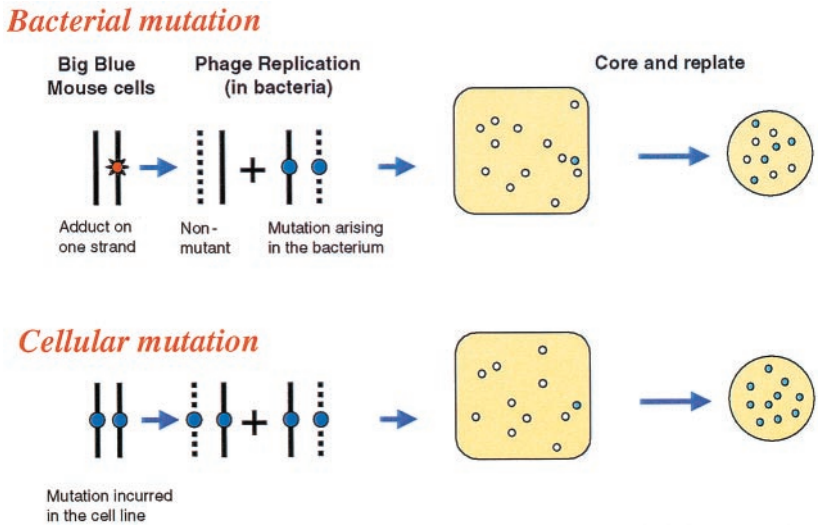

Fig. 1. The generation of mosaic and homogenous mutant plaques. In the first example (*Upper*), a lesion created within the cell line forms a mutation in the bacterium during phage replication. A lesion fixed into a mutation during the first round of phage replication produces a mosaic plaque containing a 1:1 ratio of mutant to wild-type phage, as indicated after replating the plaque. In the second example (*Lower*), a mutation sustained within the cell line leads to the formation of a homogenous mutant plaque, which is also made evident on replating.

tiple copies, approximately 40, of the Big Blue λ shuttle vector located on chromosome 4 (7). This vector contains the *lacI* transgene as a mutational target. The λ/*lacI* vector is roughly 45 kb and is identical to that of the Big Blue transgenic mouse. This vector can be easily recovered with the use of *in vitro* packaging extract; mutations at the *lacI* transgene are detected as described below.

Cell Culture. Big Blue mouse cells were cultured in DMEM (GIBCO/BRL) containing 10% (vol/vol) FBS (GIBCO), 1% L-glutamine (GIBCO), and 1% penicillin/streptomycin (GIBCO). A culture atmosphere of 5–7% CO_2 was maintained in a humidified incubator at 37°C. Stock cultures were grown in 100-mm tissue culture dishes (Sarstedt, PQ, Canada).

To induce quiescence, cultures were seeded in 100-mm dishes (Sarstedt) with 1×10^6 cells in the presence of serum and incubated overnight. After a 24-h growth period, the cultures were washed three times with PBS (pH 7) and reincubated with serum-free medium (DMEM, 1% L-glutamine, and 1% penicillin/streptomycin). The serum-free medium was renewed at 4-day intervals. Quiescent cells were transferred from 100-mm culture dishes to 150-mm dishes and subsequently cultured under standard conditions to induce cell proliferation.

Mutagen Treatment. All cultures were prepared by seeding 1×10^6 cells in 100-mm culture dishes and incubating under standard growth conditions. Proliferating (24-h after plating) and quiescent (12 days after serum removal) cultures were treated with ENU (200 μg/ml; Sigma; CAS no. 759-79-9). In a subsequent experiment, an ENU concentration of 400 μg/ml was used to treat quiescent cells that were later induced to proliferate to elucidate the mutational kinetics more accurately on restart. All mutagen treatments were conducted for 30 min in serum-free media at standard cell culture conditions (see above). Cultures then were washed three times with PBS. Quiescent and proliferating cells later were incubated in serum-free or standard medium, respectively. Thereafter, cells were trypsinized (GIBCO), spun down (1,000 × *g*), and quick-frozen with liquid nitrogen at 0, 1, 2, 3, and 4 days after incubation and stored at −80°C until further analysis.

Cell Division and DNA Synthesis Determination. Cells were detached with trypsin and counted by using a hemocytometer (Exacta, Dresden, Germany). The incorporation of [*methyl*-^{3}H]thymidine (Amersham Pharmacia) into DNA was used as an indicator of DNA synthesis after incubation [1 h, at 37°C with 1 μCi/ml at a specific activity of 75 Ci/mmol (1 Ci = 37 GBq)]. DNA was isolated from pulse-treated cells and resuspended in Tris/EDTA buffer, as described below. The radioactivity of the DNA was quantified with a Packard liquid scintillation analyzer (model TR 1600TR).

Cell Survival. Cells were detached daily with trypsin and 200 cells were seeded in each of four 100-mm culture dishes in the presence of serum. After incubating the cultures for 10 days, the relative survival of the serum-starved cells to the proliferating cells was determined by counting and comparing the resulting colonies after staining with 4% (wt/vol) Giemsa.

DNA Isolation. Big Blue mouse cells were thawed and then resuspended in proteinase K solution (2 mg/ml; Sigma; CAS no. 39450-01-6). Genomic DNA was purified from the cell suspension after an incubation time of 2 h at 55°C, followed by phenol:chloroform (1:1) extraction and precipitation with ethanol as described in ref. 8. The precipitated DNA was spooled onto a hooked glass Pasteur pipet, air dried, and dissolved in 50 μl of Tris/EDTA buffer (10 mM Tris/4 mM EDTA, pH 8). The DNA was allowed to dissolve during an overnight incubation at room temperature. The concentration of DNA then was determined spectrophotometrically after diluting the DNA solution 1:100 in Tris/EDTA (Beckman Coulter; general purpose UV-visible model DU 520) at 260 nm.

O^6-Alkylguanine-DNA Alkyltransferase (AGT) Assay. The activity of AGT in cell extracts was measured by the transfer of the [^{3}H]methyl adduct in [^{3}H]methyl DNA alkylated with [^{3}H]*N*-methylnitrosourea as previously described (9). The assay for alkyltransferase was modified slightly from a previously described method as outlined below (10). Cell extracts from quiescent and proliferating cells were prepared as previously described (10). Protein (400 μg) from these extracts was combined with 15 μg of [^{3}H]methyl DNA in the assay buffer (70 mM

Hepes, pH 7.8/0.1 mM EDTA/5% (vol/vol) glycerol/1 mM DTT/25 μM spermidine) in a final volume of 200 μl and incubated for 2 h at 37°C. The reaction was stopped with 40 μl of 50% trichloroacetic acid and incubated at 4°C for 30 min. After centrifugation at 10,000 × g, the pellet was washed twice with 95% (vol/vol) ethanol, hydrolyzed at 80°C for 1 h with 80 μl of 0.1 M HCl, and then neutralized with 400 μl of 0.02 M Tris base (pH 10.6). Hydrolyzed purines present in the supernatant were separated by HPLC through an Applied Biosystems C8 column (220 × 4.0 mm) equipped with a guard column by elution at 1.0 ml/min with a gradient of 100% 0.01 M KH_2PO_4, pH 6.2, to 75% 0.01 M KH_2PO_4/25% methanol over 25 min as previously described (11). The O^6-methylguanine removed was calculated from the difference between the radioactivity (cpm) detected within the O^6-methylguanine fraction in the control and experimental assays. The control assay substituted denatured proteins from the equivalent cell extract used for the corresponding experimental assay.

DNA Packaging/*lacI* Assay. Transpack (Stratagene) packaging extracts were used to package and screen [on a bacterial lawn (strain SCS-8) in the presence of 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal)] isolated DNA for *lacI* mutants according to the manufacture's recommendations, with the following changes: (i) the packaged DNA samples first were titered to ensure that fewer than 5,000 plaques formed on each assay tray (Stratagene), and (*ii*) the top X-gal-enriched layer of medium was supplemented with 10 g/l of agarose to decrease phage migration.

Determination of Mutant Plaque Mosaicism. Well isolated (>4 mm apart) mutant plaques were cored with a 200-μl pipet tip and resuspended in 500 μl of phage buffer with 50 μl of chloroform (Caledon Laboratories Ltd., ON, Canada) and stored at 4°C as recommended by Stratagene. The next day the phage were adsorbed in bacterial suspension for 30 min at 37°C and screened for mosaicism after overnight plate incubation. Plaques containing ≥90% mutant phage were classified as cellular mutations, whereas plaques that contained ≤50% mutant phage were classified as bacterial mutations. No ambiguous plaques (50%–90% mutant) were recovered.

Results

Cellular Division and DNA Replication. To investigate the role of DNA replication on mutation induction, it was necessary to maintain Big Blue mouse cells in a division-arrested state. Big Blue (1×10^6) mouse cells were seeded in 100-mm tissue culture dishes (≈80% confluency) in the presence of serum-supplemented medium. Fig. 2 depicts the effect of serum deprivation and contact inhibition on cellular division. Switching Big Blue mouse cells from serum-containing medium to serum-free medium immediately affects the division rate: No significant increase in cell number was observed throughout the 16 days of serum-free culture. DNA synthesis, as measured by the incorporation of tritium-labeled thymidine into high molecular weight DNA, does not cease immediately, but rather declines exponentially over time ($y = 165e^{-0.6x}$, $R^2 = 0.96$; Fig. 3). After 12 days of serum deprivation, the level of DNA synthesis drops to below 2% of normal and to less than 0.5% by day 16.

Cell Survival. Because the possibility exists for increased cell death during serum-free incubation, it was essential to determine the relative viability of serum-deprived cells, as it could explain the continuation of DNA synthesis in the absence of a change in cell number. Cultures used during this study maintained a 70–100% absolute cloning efficiency during the 16 days of serum deprivation (Fig. 4). There was a slight decline (although not significant) in cloning efficiency during the period of quiescence in serum-free medium, but this decline was not correlated with the observed residual rate of DNA synthesis.

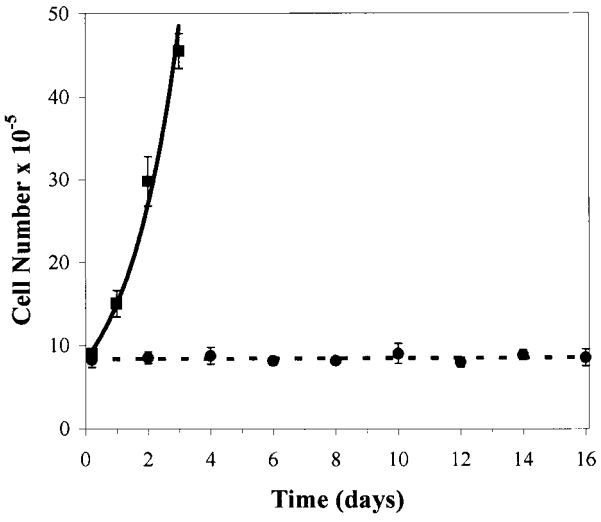

Fig. 2. The effect of serum-free medium on cell division. Control cultures (■) are growing in medium supplemented with 10% (vol/vol) FBS, whereas cells deprived of serum (●) remain quiescent. Cells were counted with a hemocytometer after being removed with trypsin (±SD).

Mutation in Proliferating and Arrested Cultures. To elucidate the relationship between proliferation and mutation, both arrested and exponentially growing cells were treated with ENU in serum-free medium for 30 min. A relatively low dose of ENU (200 μg/ml of ENU in serum-free medium for 30 min at 37°C) was chosen for this study because it did not affect DNA synthesis, cell division, or cell viability in either quiescent or exponentially growing cultures (data not shown). The acute dose of ENU was administered to both quiescent and proliferating cells. Proliferating cells were treating during their exponential growth period,

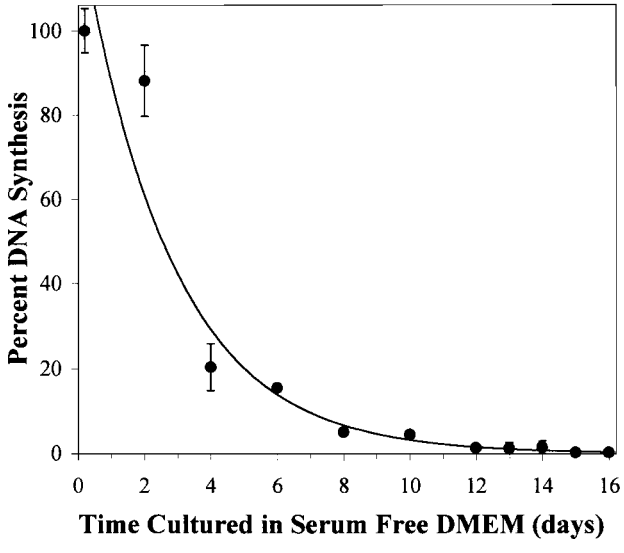

Fig. 3. The effect of serum deprivation on DNA synthesis. DNA synthesis was determined after incorporation of [*methyl*-^{3}H]thymidine into DNA (±SD) during a 1-h incubation (1 μCi/ml). DNA synthesis is expressed relative to that of the exponentially growing control cultures at zero time.

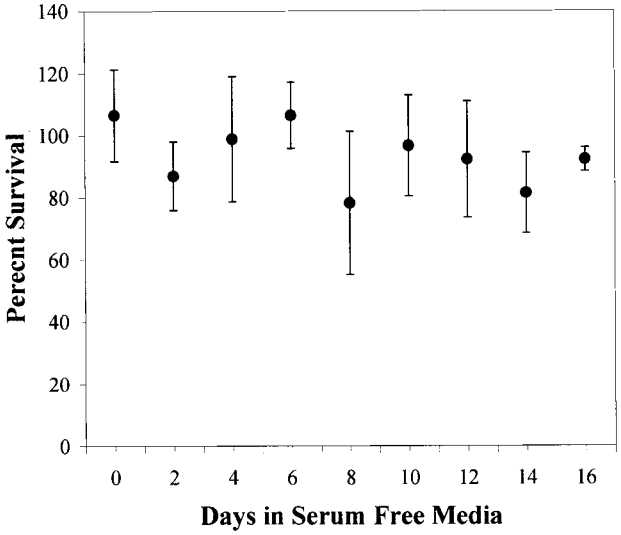

Fig. 4. Relative survival of Big Blue mouse cells after serum deprivation as measured by absolute cloning ability (±SE).

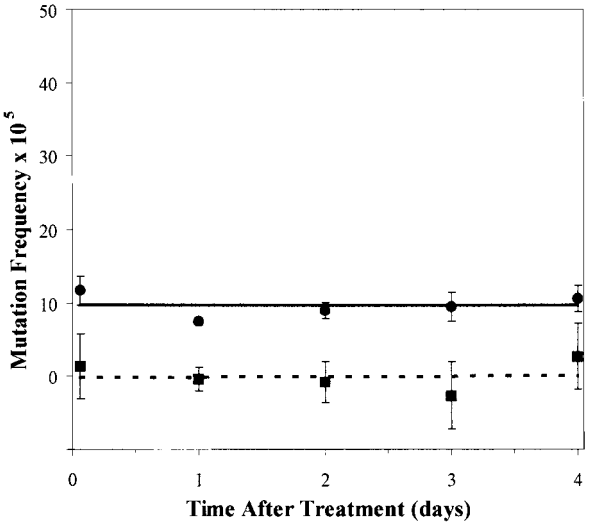

Fig. 6. Adduct stability in quiescent cultures. The frequency (±SD) of bacterial (●) and cellular (■) *lacI* mutations in quiescent Big Blue mouse cells vs. time. Quiescent cultures (12 days after serum removal) were treated with ENU (200 μg/ml) in serum-free medium for 30 min at standard cell culture conditions.

whereas the quiescent cultures were treated after 12 days of serum deprivation.

Initially, bacterial and cellular mutations are indistinguishable from one another as they both form blue plaques (6). However, coring and replating the plaque reveals the method by which the mutation was created. Immediately after treatment, all induced mutations were bacterial in both proliferating (Fig. 5) and division-arrested cultures (Fig. 6). Exponentially growing cultures showed a 5-fold higher mutation induction than their quiescent counterparts, although this difference is noteworthy, the explanation for this phenomenon is not clear at present. In proliferating cells, bacterial mutations decreased with time, coincident with an accumulation of cellular mutations (Fig. 5). In contrast, the frequencies of bacterial and cellular mutations in quiescent cells remained constant over time (Fig. 6). When quiescent cells were induced to proliferate, repair and cellular mutation induction followed (Fig. 7). Roughly half of all of the induced bacterial mutations in quiescent cells were transformed into cellular mutations subsequent to induced proliferation. Proliferating and quiescent untreated control cultures have similar spontaneous mutant frequencies, $24.1 \pm 2.6 \times 10^{-5}$ and $25.0 \pm 3.1 \times 10^{-5}$ respectively. Of the spontaneous *lacI* mutations that were recovered, less than 0.5% was bacterial in origin.

Although the decrease of bacterial mutations in proliferating and proliferation-stimulated cultures is associated with a rise in cellular mutations, it should be noted that not all of the DNA adducts are transformed into mutations within the cell line (Fig. 5). These results show that adducts at the *lacI* transgene are

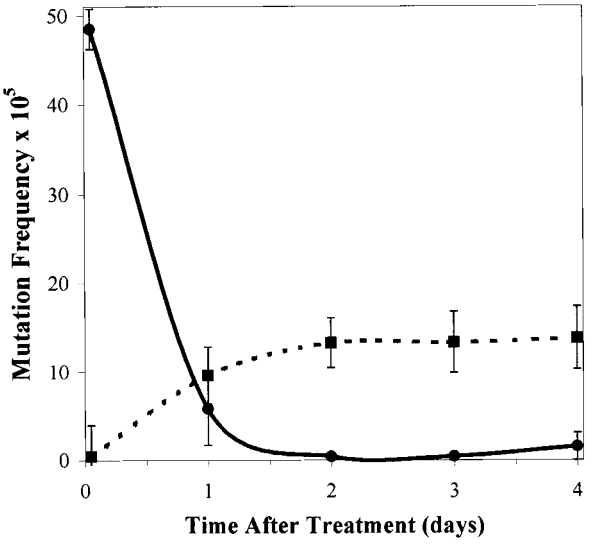

Fig. 5. The kinetics of repair and mutation in proliferating mouse cells. The frequency (±SD) of bacterial (●) and cellular (■) *lacI* mutations in proliferating Big Blue mouse cells was measured as a function of time after mutagen (ENU) treatment.

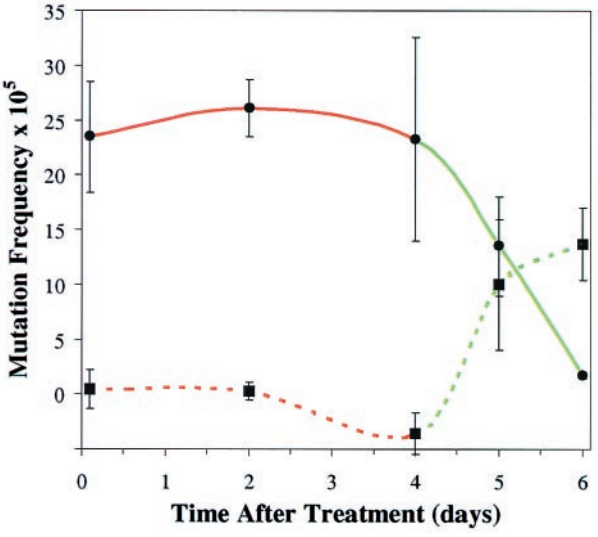

Fig. 7. Mutation and repair kinetics in arrested cell cultures induced to proliferate. The frequency (±SD) of bacterial (●) and cellular (■) *lacI* mutations in quiescent cultures (12 days after serum removal) treated with ENU (400 μg/ml) in serum-free medium for 30 min at standard cell culture conditions. Quiescent cells (red) were induced to proliferate 4 days posttreatment (green). Mutation frequencies less than zero arise when an observed value in the treated group is less than that in the controls.

being fixed and repaired in proliferating cells: Quiescent cells show no sign of adduct repair or fixation but rather demonstrate adduct stability.

Discussion

The role of cell proliferation on mutation, and the associated risk of carcinogenesis, has been actively debated (1,12–16). Although it remains clear that cell proliferation plays an important role in mutation, the data provided to date do not provide critical evidence for its requirement in mutation (17). What has been lacking in previous studies has been achieved in this one with the use of the transgenic Big Blue mouse cell line, namely, proliferation as the only independent variable. When deprived of serum during confluence, these mouse cells quickly enter quiescence. Although cellular division immediately ceases on serum withdrawal, DNA synthesis continues. This disparity could have resulted from an increase in cell death during the initial few days of serum deprivation, which subsequently leads to an increase in cell division as the dead cells are replaced. This clearly is not the case, as the relative survival remains steady at approximately 80% during this time interval. During ENU treatment, the level of DNA synthesis is below 2% relative to control cultures, thus this initial disparity observed between the rate of cell division and DNA synthesis during the first few days of serum deprivation remains only a minor factor in these experiments.

After ENU treatment, all induced *lacI* mutations were initially bacterial in both proliferating (Fig. 5) and division-arrested cultures (Fig. 6). Because bacterial mutations are representative of adducts at the *lacI* transgene, this result is expected, given that no time has elapsed to permit repair or mutation fixation. The same ENU dose, however, initially inflicted 5 times more premutagenic damage in proliferating cultures than in their quiescent counterparts, as shown by the frequency of bacterial mutations. This finding could be a consequence of an increase in ENU transport into proliferating cells, or of the elevated number of damage-sensitive unwound templates exposed in proliferating cells during DNA replication (18), or of a number of as-yet-unknown factors that may protect quiescent cells from premutagenic DNA damage.

Because the cellular mutant frequency (4 days posttreatment) is 27% of the initial bacterial mutant frequency (Fig. 5), a maximum of 27% of the ENU-induced lesions was converted into mutations within the mouse cells in 4 days. This is a maximum because there may be lesions that are mutagenic in the murine cells but not in the bacteria. Similarly, a minimum of 73% of the lesions present in the mouse cell was not converted to cellular mutation and thus was repaired. In contrast, the original level of DNA lesions observed in quiescent cells remained unchanged for 4 days posttreatment, showing no sign of repair or conversion into cellular mutations. Both repair and mutation fixation ensued once quiescent cells were induced to proliferate. Clearly, the data indicate that proliferation is a requirement for both repair and mutation resulting from ENU-induced damage in the Big Blue mouse cell line.

The decline in the frequency of bacterial mutations and a concomitant increase in cellular mutations over the first cell cycle after mutagenesis could arise in two ways: The lesions could be diluted as a result of DNA replication or they could be eliminated by DNA repair. In one cell cycle, the dilution of lesions in the absence of DNA repair would be 2-fold, yet we observe an almost complete loss of bacterial mutations, so DNA repair is obviously the dominant factor. The rise in cellular mutations during the same period could be the result of replication past an unrepaired lesion or the misrepair of a lesion. Because all cellular mutations arose in approximately one cell cycle, coupled with the simultaneous decline of bacterial mutations, it is tempting to assume that the lesions giving rise to cellular mutations are the same as those giving rise to bacterial mutations. If so, roughly one-fourth of the lesions that give rise to bacterial mutations produce mutations in the mouse, because there are initially about 4 times as many bacterial mutations as there are cellular mutations. Unfortunately, all mutagens produce a variety of DNA lesions, so it is possible that the lesions that give rise to the mutations in the mouse cell and those that give rise to bacterial mutations are not identical. The extensive array of bacterial DNA repair mutants can be used to analyze the repair mechanisms in mouse cells. The underlying concept is identical to that already described (18): Bacterial mutations represent lesions induced in the DNA of the mouse cells that are then converted into mutations in the bacteria. Clearly the efficiency of the formation of these mutations will depend on the repair capabilities of the bacteria. Thus, by using bacteria with differing repair capacities whose specificity for DNA lesions is known, it should be possible, in the future, to discriminate among the DNA lesions in the mouse cell and subsequently measure their rates of repair.

Given that a large burden of repair of ENU-induced adducts falls on the repair protein AGT, one might expect this protein to be absent or dramatically reduced in the repair-deficient quiescent cells. The AGT activity (per microgram of protein in the cell lysate) in quiescent and proliferating cells was determined to be 12.0 ± 1.4 cpm and 15.9 ± 0.1 of O^6-methylguanine transferred, respectively. Surprisingly, the activity of AGT is only reduced by 25% in quiescent cells when compared with their actively dividing counterparts, which alone cannot account for the absence of repair observed in quiescent cells. Thus, these data provide evidence for a proliferation-induced repair system, where proliferation or its initiation may provide the signal essential for the induction and activation of DNA repair.

Our current data indicate that neither mutation nor repair occurs in quiescent cells. The lack of repair in quiescent cells, despite the existence of a very significant level of AGT, is very surprising and is at variance with one of the dominant themes in the somatic mutation theory of cancer, the importance of the rate of proliferation. One has only to look at the reaction to Farber's suggestion that cellular proliferation is not important in carcinogenesis (17) to see how entrenched this idea is and how many arguments there are in its favor, for example, the reaction by Ames and Gold (17). Those who believe that promoters act by increasing cellular proliferation often cite a decreased time available for DNA repair as one of the mechanisms (19). If the cells do not repair unless they are proliferating, then this explanation cannot be correct. Our results do show that the amount of damage experienced by the cell is very substantially reduced under nonproliferating conditions. At this point it is uncertain whether this is a peculiarity of this cell line, this mutagen (ENU), or the means by which quiescence was induced. We note that these cells can repair premutagenic DNA damage when they are proliferating or are induced to proliferate.

We have adapted the Big Blue system to allow for the dynamic qualification of DNA lesions and mutations at the *lacI* transgene, providing the means to study mutation fixation, adduct stability, and repair in quiescent and proliferating Big Blue cells. There seems no reason why this system cannot be used to elucidate the kinetics of repair and mutagenesis of a variety of mutagens in both proliferating and quiescent cells.

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